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Prenatal and Molecular Diagnosis of Hemophilia B

Ji-Hsiung Young, Jyh-Chwan Wang, Jyh-Pyng Gau, and Han-Tien Hu
Section of Hematology, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan

Prenatal diagnosis was carried out on a woman who had previously given birth to a son with a spontaneous mutation of C → T transition at nt 31133 of the factor IX (F.IX) gene. The diagnosis was performed on chorionic villi sampling by the method of amplification-created restriction site (ACRS). It revealed a female fetus with a normal F.IX gene, as confirmed by DNA sequencing after delivery. Meanwhile, a survey using the ACRS method to evaluate the inheritance of 63 individuals from 8 hemophilia B families was done. A different single-point mutation in each family was proved by DNA sequencing. One individual had a mutation with a naturally-created restriction site. In each of the remaining patients, we were able to show an enzyme-cutting site in their DNA amplification product for ACRS with the designed mutagenesis primers. All patients and carriers could be diagnosed accurately by comparing ACRS results with clinical and laboratory findings. There were new novel mutations among the patients. © 1996 Wiley-Liss, Inc.

Key words: hemophilia B, factor IX, mutation, prenatal diagnosis

INTRODUCTION

With presymptomatic DNA analysis and feasible gene therapy possible in the future, prenatal diagnosis is one of the major works in hemophiliac care. Hemophilia B is caused by the deficiency of coagulation factor IX (F.IX). Carrier detection and prenatal diagnosis have been based on the molecular analysis of proteins. But this has not been practical in genetic counseling, especially for carrier detection. The gene regulating F.IX synthesis is located in the long arm of the X chromosome, in q27.1 [1–3]. It spans eight exons and seven introns [4–6]. Up to now, more than 1,000 unique molecular events of F.IX gene mutation, including insertion, deletion, and point mutation, have been reported [7,8]. However, no specific spectrum of mutation of the gene has been defined as a common pattern in any population, as with thalassemia [9]. Although several procedures have been developed for hunting gene mutations, there are still limitations of rapidity, accuracy, and simplicity.

Amplification-created restriction site (ACRS) is a PCR-based method that can evaluate a gene mutation by means of a designed mutagenesis primer [10,11]. The primer is constructed by terminal base modification according to the nucleotide arrangement at the mutation point, so as to create or abolish a restriction site to the point on the amplified products of either a normal or a mutant gene. After digesting the products with a corresponding endonuclease, the normal gene can be distinguished from the mutant simply by directly visualizing the sizes of the digested fragments on an electrophoresis gel. Basically, ACRS provides an accurate and rapid procedure for detecting mutated genes without requiring any radioactive material as in some other procedures. In this study, the ACRS method was applied to diagnose the health status of a fetus. We hope that ACRS will be more widely applicable in prenatal diagnosis; we have done a familial survey to detect individuals, both carriers and patients, inheriting a mutant gene of familial significance.

MATERIALS AND METHODS

Patients

The pregnant woman, G3P3, was healthy, but her son was shown to have hemophilia B caused by spontaneous mutation. She received a prenatal diagnosis in her tenth week of pregnancy. In addition, 63 individuals from another 8 nonconsanguineous families, including 13 patients and 22 carriers, were also entered into the study to assess

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Address reprint requests to Ji-Hsiung Young, M.D., Section of Hematology, Department of Internal Medicine, Taichung Veterans General Hospital 160, Section 3, Chung-Kan Road, Taichung, 407, Taiwan.

TABLE I. Characteristics of Primers Designed for PCR and DNA Sequencing*

Primers	Sequences	Amplification		
		nt, position	DNA length	Conditions (°C)
F91A52	5′-TTCAGACTCAAATCAGCCACAG-3′	−193–−172	456 bp	94, 53, 72
F91A3	5′-TACTTACCAACCTGCGTGC-3′	162–144		
F94D52	5′-CATCCCAATGAGTATCTACAGG-3′	10310–10331	433 bp	94, 51, 72
F94D32	5′-TAATAAGGTGAGTCGGAACATC-3′	10721–10742		
F95E5	5′-GACCCATACATGAGTCAGTAGTTCC-3′	17584–17608	317 bp	94, 53, 72
F95E3	5′-AAAAGGAAGCAGATTCAAGTAGG-3′	17900–17878		
F96F53	5′-TCTCAGAAGTGACAAGGATGG-3′	20260–20280	382 bp	94, 53, 72
F96F3	5′AATAGCCTCAGTCTCCCACC-3′	20641–20622		
F98b5	5′-TGGCTATGTAAGTGGCTGG-3′	31033–31052	192 bp	94, 55, 72
F98a32	5′-GGTCCCCCACTATCTCCTTG-3′	31205–31224		

*The number of the third character in the described primers denotes the corresponded exon of F.IX gene; F9″1″.
nt, nucleotide.

the accuracy and rapidity of detecting those who carried a mutant gene. A detailed family history of hemorrhage and a complete coagulation survey was required. The coagulation tests included prothrombin time (PT), activated partial thromboplastin time (APTT), PT- or APTT-based assay for each factor, and detection of F.IX inhibitor [12]. The F.IX antigen level was evaluated by rocket immunoelectrophoresis.

Direct DNA Sequencing

High molecular weight DNA was prepared from the chorionic villi sampling, as were peripheral blood leukocytes according to the method of Maniatis et al. [13]. DNA was recovered by phenol-chloroform extraction and then ethanol precipitation. DNA was stored for sequencing.

To proceed to the direct sequencing, DNA amplification for all exons was done using a Gene Amp PCR kit and an automatic DNA Thermal Cycler-480 (Perkin Erlmer Cetus). The amplified products were purified from agarose gel and subjected to direct nucleotide sequencing using the dsDNA cycle sequencing system (Gibco, BRL), following the recommendations of the supplier. Same pairs of primers were used for DNA amplification and sequencing by the dideoxy chain-termination method described by Sanger et al. [14]. The structures and reaction conditions of the primers are shown in Table I.

Human Lymphocyte Antigen (HLA) and Blood Typing

PCR amplification with sequence-specific primers (PCR-SSP) using oligonucleotide primers was applied to examine the allelic polymorphism of DRB1 and DQB1 for the parenthood study of H349 according to Olerup and Zettergaise [15] and Olerup et al. [16]. Blood grouping was also performed.

Design of Mutagenesis Primers

For evaluation of the mutant genes without forming a naturally-created cutting site, the mutagenesis primers, either as 3′- or 5′-end primers, were constructed by terminal base modification according to nucleotide sequence. Hence, a restriction site recognized by a corresponding endonuclease was artificially created for each amplified product. The characteristics, PCR conditions, and paired primers for ACRS are shown in Table II.

Prenatal Diagnosis by ACRS

Each DNA sample was amplified by one pair of primers, including a mutagenesis primer, using the Gene Amp PCR kit (Perkin Erlmer Cetus), and following the procedures described for direct DNA sequencing. On completing the reaction, each PCR-product was digested with a selected endonuclease and followed by electrophoresis on a 3% or 4% agarose gel, depending on the size of the digested fragments predicted. Results were interpreted directly on the gel and/or on a photograph.

RESULTS

Based on clinical manifestations, family history, and results of coagulation studies, the definite diagnosis of hemophilia B was made. The pregnant woman, her husband, and her daughter were proved to have no abnormality in both F.IX:C and F.IX:Ag. But her son, patient 349, was found to have hemophilia B with F.IX:C $< 1\%$ and F.IX:Ag $< 1\%$. He was suspected of having an abnormal F.IX gene caused by spontaneous mutation. Therefore, we first evaluated their parenthood. We examined blood grouping and HLA-DNA (DR and DQB1) typing. It was shown that they belonged to type O, except for type B in the son and his sister. The DRB1 and the DQB1 alleles studies showed a good correlation of sanguineous relationship.

PCR followed by DNA sequencing for patient 349 revealed that he had a point mutation of C → T at nt 31133, as shown in Table II. In addition, there was a Taq I restriction site, TCGA, on the germline sequence, but it was lost when the mutation, TTGA, arose on the site. For rapid survey and antenatal diagnosis with the ACRS

TABLE II. Structures, Chemical Characteristics, and Corresponding Endonucleases to the Primers Designed for Amplification-Created Restriction Site (ACRS)

Primers	Sequences	Amplified DNA, length (bp), and nature		
		Length/enzyme (site)	Digested product	Conditions (°C)
PH41	CCA ATT CAA TTT CTT AAC CTA TCT CAA gGA T	380 bp/FokI (GGATG)	N: 288+51+41	95, 1′; 53, 1′; 72, 1′
F94D32	TAA TAA GGT GAG TCG GAA CAT C		M: 288+92	
F98H5	GCC AAT TAG GTC AGT GGT CC	388 bp/ScaI (AGTACT)	N: 365+23	95, 1′; 51, 1′;72, 1
PH51	GTC AAC AAG TGG AAC TCT AAA GTA C		M: 388	
F94D52	CAT CCC AAT GAG TAT CTA CAG G	169 bp/StyI (CCTAGG)	N: 146+23	95, 1′; 53, 1′; 72, 1′
PH211	CAA AGG GAC ACC AAC ATT Cc		M: 169	
F95E5	GAC CCA TAC ATG AGT CAG TAG TTC C-3′	111 bp/BglII (AGATCT)	N: 84+27	95, 1′; 53, 1′; 72, 1′
PH235	GCC ATT CTT AAT GTT ACA TGT TAg AT-3′		M: 111	
F95E5	GAC CCA TAC ATG AGT CAG TAG TTC C	151 bp/AluI (AGCT)	N: 123+28	95, 1′; 53, 1′; 72, 1′
PH267	CCT TGT TAT CAG CAC TAT TTT TAC AAA g		M: 151	
F91A52	TTC AGA CTC AAA TCA GCC ACA G	456 bp/AluI (AGCT)	N: 291+160	95, 1′; 53, 1′; 72, 1′
F91A3	TAC TTA CCA ACC TGC GTG C		M: 160+146+145	
F96F53	TCT CAG AAG TGA CAA GGA TGG	138 bp/StyI (CCTAGG)	N: 112+26	95, 1′; 55, 1′; 72, 1′
PH294	TTG TGA AAC AGA AAC TCT TCC c		M: 138	

bp, base pairs; N, normal; M, mutation.

TABLE III. Laboratory Results of Coagulation Tests, Change of Amino Acid, and Locations of F.IX Gene Mutation in 9 Patients With Hemophilia B*

Patient	Mutation	AA change and comments	Position		F.IX:C (%)	F.IX:Ag (%)	Inhibitor
			nt	Exon/IVS			
H293	G→C[a]	Donor splice, first base	118	IVS-1	<1	<1	−
H41	G→A	Gly(48)→Arg	10394	Exon d	25	77	−
H368	G→A	Gly(48)→Arg	10394	Exon d	32	70	−
H211	A→C[a]	Tyr(69)→Ser	10458	Exon d	<1	73	−
H235	G→T[a]	Acceptor splice, first base	17668	IVS-4	<1	<1	−
H267	ΔG	Frameshift	17706	Exon e	<1	<1	−
H51	A→C	Gln(324)→Pro	31092	Exon h	<1	<1	+
H294	G→A	Cys(132)→Tyr	20375	Exon f	<1	<1	−
H349	C→T	Arg(338)→Stop	31133	Exon h	<1	<1	−

*AA, amino acid; nt, nucleotide.
[a]Novel mutations.

procedure, we first amplified the DNA sequence near the mutant point with the paired primers, F98H52 and F98H32. A product, 711 bp in length, was obtained. The product released three fragments, of 142, 258, and 311 bp, by Taq I digestion. However, the mutant gene became only two fragments 142 and 569 bp long. The result of rapid diagnosis by ACRS procedure for the DNA prepared from chorionic villi sampling was exactly as predicted (Fig. 1).

Results of the coagulation study and the locations of F.IX gene mutations in the other 8 families are summarized in Table III. Severe hemophilia B was found in 6 of them. A different single-point mutation in each family was localized by sequencing the entire coding regions and also their adjacent portions in each neighboring intron. Three of the patients, H41, H368, and H211, had normal F.IX:Ag and a gene mutation in exon d. Their F.IX:C was undetectable in one and reduced in the other two. The remaining patients had marked deficiencies of both activity and antigen of F.IX. Patient H293 had a mutation at nt 118 in the donor-splicing junction of exon a. It naturally created an Alu I-recognized site as shown in Table III. We could create different restriction sites artificially for the other seven patients by designed mutagenesis primers for PCR procedures and by rapid survey. The mutant point was constructed at either the 3′- or 5′-end of the products. Therefore, the fragments with predicted lengths were obtained after digestion with the corresponding enzyme, as shown in Table II.

DISCUSSION

Although the incidence of hemophilia B is low, the data collection of F.IX gene mutations has displayed more than 800 patterns of molecular anomaly [7]. In addition, there is a great variation of gene defects among ethnic groups. With recent progress in molecular genetics, the possibility of detecting a defective gene in an individual

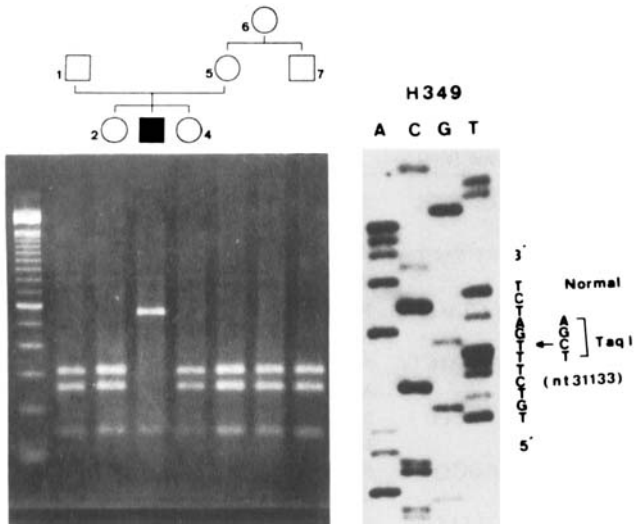

Fig. 1. Family pedigree, ACRS findings (left), and F.IX gene mutation of patient H349 and his family (right). ACRS shows findings of DNA amplification with primers F98H52 and F98H32, followed by digestion with TaqI. Results show that only H349 (lane 4) had a point mutation, resulting in an abnormal fragment of 569 bp, while chorionic villa sampling (lane 5) and other family members with the normal F.IX gene had fragments of 311 and 258 bp.

has been greatly increased. Several methods have been developed and applied in searching for mutant genes, including: intragenic or extragenic polymorphism [17–20], DGGE (denaturing gradient gel electrophoresis), allele-specific oligonucleotide (ASO) probes [21], allele-specific PCR [22], direct DNA sequencing, single-strand DNA conformational polymorphism (SSCP) [23], and other PCR-based methods [24]. But there are still some limitations to their application in the massive survey of F.IX gene defect.

About one third of cases of hemophilia B are caused by de novo mutation of the germline gene [7]. However, H349 is the only patient with a spontaneous mutation in our registration. The blood grouping shows that both his mother and sister are B-phenotype, while the other members of his family are all O-phenotype. Though we cannot definitely delineate their relationship simply by the test, the HLA allele studies show that he has DRB1*8 and DQB1*0601 inherited from his mother, and DRB1*9, DRB1*53, and DQB1*0303 from his father. Moreover, the patient and his father appear similar to one another. The information helps confirm their parent and son relationship.

Patient H349 has a C → T mutation at nt 31133 in exon h resulting in Arg (338) → Stop. It abolishes the normally present Taq I restriction site, TCGA. Based on this fact, prenatal diagnosis by ACRS was performed for the most recent pregnancy of this patient's mother using primers F98H52 and F98H32. The findings of chorionic villi and leukocytes of his family were compared at the same time. Only patient H349 had an abnormal band of 569 bp by Taq I digestion, as shown in lane 4 of Figure 1. On the other hand, the family, including the fetus, had normal ACRS results. The conclusion is that the fetus carries a normal F.IX gene. This was confirmed by DNA sequencing after delivery.

In this study, the different single-point mutations in the other 8 patients were ascertained by PCR followed by DNA sequencing. The significance of the mutations was confirmed by comparing all information, including a detailed family history, a complete hemostatic examination, and gene studies. The results for the 8 families are summarized in Table III. The severity of clinical features, serum levels of both F.IX:C and F.IX:Ag, and the corresponding gene mutations in 5 of our patients were similar to those in previous reports [7,25]. However, we have three novel mutations that have never been presented previously: A → C at nt 10458, G → T at nt 17668, and G → C at nt 118. The rapid screening for the individuals carrying the mutant F.IX gene of familial significance was done by ACRS. Patient H267 was a spot case. We did not do a familial study for him, because he was adopted and has had no contact with his biological parents. We accurately detected the 35 individuals, including 13 patients and 22 carriers, from the other 7 nonconsanguineous families. The study of diagnosing a person carrying a mutant gene shows that there is a better correlationship between ACRS findings and DNA sequencing than between coagulation studies and family history, especially for assessment of a carrier. Therefore, ACRS provides better carrier detection, especially for hemophilia B with mild severity in the family history. It is a very convenient and rapid procedure for detecting mutations, requiring no radioactive material, and minimizing environmental contamination. In addition, results can be interpreted simply on gels. Our results have shown that we can successfully design mutagenesis primers to all mutations for ACRS. Moreover, a mutation sometimes creates a restriction site naturally. ACRS also gave us some advantages in screening. None of our patients was failed in the detection. Selecting an appropriate gel concentration for a good separation of the digested products presented our only difficulty, but, this could be resolved easily by adjusting the gel concentration depending mainly on size of the products. ACRS is very useful in the familial survey of hemophilia B, as it also is in the diagnosis of thalassemia [9], ras oncogene mutation [26], PKU [10], and G6PD deficiency [11].

Genetic counseling always plays a very important role in a hemophilia comprehensive center. Rapid and accurate carrier detection and reliable prenatal diagnosis are necessary in controlling this inherited disease. We have been able to achieve these in our patients.

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REFERENCES

1. Camerino G, Grzeschik KH, Jaye M, De La Salle H, Tolstoshev P, Lecocq JP, Heiling R, Mandel JL: Regional localization on the human X-chromosome and polymorphism of the coagulation factor IX gene (hemophilia-B locus). Proc Natl Acad Sci USA 81:498–502, 1984.
2. Chance PF, Dyer KA, Kurachi K, Yoshitake S, Ropers HH, Wieacker P, Gartler SM: Regional localization of the human factor IX gene by molecular hybridization. Hum Genet 65:207–208, 1983.
3. Schwartz C, Fitch N, Phelan MC, Richer CK, Stevenson R: Two sisters with a distal deletion at the Xq28/Xq27 interface: DNA studies indicate that the gene locus for factor IX is present. Hum Genet 76:54–57, 1987.
4. Anson DS, Choo KH, Rees DJG, Giannelli F, Gould K, Huddleston JA, Brownlee GG: The gene structure of human anti-haemophilic factor IX. EMBO J 3:1053–1060, 1984.
5. Choo KH, Gould KG, Rees DJG, Brownlee GG: Molecular cloning of the gene for human anti-hemophilic factor IX. Nature 299:178–180, 1982.
6. Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K: Nucleotide sequence of the gene for human factor IX (anti-hemophilic factor B). Biochemistry 24:3736–3750, 1985.
7. Giannelli F, Green PM, Sommer SS, Lillicrap DP, Ludwig M, Schwaab R, Reitsma PH, Goossens M, Yoshioka A, Brownlee GG: Haemophilia B: Database of point mutations and short additions and deletions, fourth edition, 1994. Nucleic Acids Res 22:3534–3546, 1994.
8. Thompson AR: Molecular biology of the hemophilas. Prog Hematol 10:195–214, 1991.
9. Chang JG, Chen PH, Chiou SS, Lee LS, Perng LI, Liu TC: Rapid diagnosis of beta-thalassemia mutation in Chinese by naturally and amplified created restriction sites. Blood 80:2092–2096, 1992.
10. Eiken H, Odland E, Boman H, Skjelkvale L, Engebretsen L, Apold J: Application of natural and amplification created restriction sites for the diagnosis of PKU maturation. Nucleic Acids Res 19:1427–1430, 1991.
11. Chang JG, Chiou SS, Perng LI, Chen TC, Liu TC, Lee LS, Chen PH, Tang TK: Molecular characterization of glucose-6-phosphatase dehydrogenase (G6PD) deficiency by natural and amplification created restriction sites: Five mutations account for most G6PD deficiency case in Taiwan. Blood 80:1079–1082, 1992.
12. Bertina RM, van der Linden: Detection and classification in molecular variants of factor IX. In Bloom AL (ed); "The Hemophilias, Methods in Hematology," Vol. 5. Churchill Livingstone, 1982, pp. 137–155.
13. Maniatis T, Fritsch EF, Sambrook J: "Molecular Cloning." New York: Cold Spring Harbor Laboratory Press, 1982, pp 458–459.
14. Sanger F, Nicklen S, Coulson AR: DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467, 1977.
15. Olerup O, Zetterquist H: HLA-DR typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours: An alternative to serological DR typing in clinical practice including donor-recipient matching in cadaveric transplantation. Tissue Antigens 39:225–235, 1992.
16. Olerup O, Aldener A, Fogdell A: HLA-DQB1 and -DQA1 typing by PCR amplification with sequence-specific primers (PCR-SSP) in 2 hours. Tissue Antigens 41:119–134, 1993.
17. Giannelli F, Choo KH, Winship PR, Rizza GR, Anson DS, Rees DJG, Ferrari N, Brownlee GG: Characterization and use of an intragenic polymorphic marker for detection of carriers of hemophilia B (factor IX deficiency). Lancet 1:239–241, 1984.
18. Lillicrap DP, White BN, Holden JJ, Giles AR: Carrier detection in the hemophilias. Am J Hematol 26:285–296, 1987.
19. Tagliavacca L, Sacchi E, Mannucci PM: Carrier detection and feasibility of prenatal diagnosis of hemophilia B by multiplex polymerase chain reaction. Int J Clin Lab Res 23:169–171, 1993.
20. Tanimoto M: Extragenic factor IX gene RFLP is useful for detecting carriers of Japanese hemophilia-B. Nippon Ketsueki Gakkai Zasshi 52:774–777, 1989.
21. Saiki RK, Bugawan TL, Horn GT, Mullis KB, Erlich HA: Analysis of enzymatically amplified beta-globulin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes. Nature 324:163–166, 1986.
22. Wu DY, Ugozzoli L, Pal BK, Wallace RB: Allele-specific enzymatical amplification of beta-globulin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757–2760, 1989.
23. Lin SW, Shen MC: Genetic basis and carrier detection of hemophilia B of Chinese origin. Thromb Haemost 69:247–252, 1993.
24. Reiss J, Neufeldt U, Wieland K, Zoll B: Diagnosis of haemophilia B using the polymerase chain reaction. Blut 60:31–36, 1990.
25. Rees DJG, Rizza CR, Brownlee GG: Haemophilia B caused by a point mutation in a donor splice junction of the human factor IX gene. Nature 316:643–645, 1985.
26. Haliassos A, Chomel JC, Tesson L, Baudis M, Kruh J, Kaplan JC, Kitzis A: Modification of enzymatically amplified DNA for the detection of point mutation. Nucleic Acids Res 17:3606, 1989.